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Effect of phosphatidylinositol and inside-out erythrocyte vesicles on autolysis of μ- and m-calpain from bovine skeletal muscle

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Abstract

The finding that phospholipid micelles lowered the Ca^{2+} concentration required for autolysis of the calpains led to a hypothesis suggesting that the calpains are translocated to the plasma membrane where they interact with phospholipids to initiate their autolysis. However, the effect of plasma membranes themselves on the Ca^{2+} concentration required for calpain autolysis has never been reported. Also, if interaction with a membrane lowers the Ca^{2+} required for autolysis, the membrane-bound-calpain must autolyze itself, because it would be the only calpain having the reduced Ca^{2+} requirement. This implies that the autolysis is an intramolecular process, although several studies have shown that autolysis of the calpains in an in vitro assay and in the absence of phospholipid is an intermolecular process. Inside-out vesicles prepared from erythrocytes had no effect on the Ca^{2+} concentration required for autolysis of either μ- or m-calpain, although phosphatidylinositol (PI) decreased the Ca^{2+} concentration required for autolysis of the same calpains. The presence of a substrate for the calpains, β-casein, reduced the rate of autolysis of both μ- and m-calpain both in the presence and in the absence of PI, suggesting that μ- and m-calpain autolysis is an intermolecular process in the presence of PI just as it is in its absence. Because IOV have no effect on the Ca^{2+} concentration required for calpain autolysis, association with the plasma membrane, at least with erythrocyte plasma membranes, does not initiate calpain autolysis by reducing the Ca^{2+} concentration required for autolysis as suggested by the membrane-activation hypothesis. Interaction with a membrane may serve to bind calpains to their substrates rather than promoting autolysis.

Keywords: Calpain; Autolysis; Protease; Calcium

1. Introduction

Both μ- and m-calpain autolyze when incubated with Ca^{2+} in an in vitro assay [1–4]. Brief periods of autolysis, from 2 to 5 min depending on the conditions, reduce the Ca^{2+} concentration required for half-maximal activity from 3–50 to 0.5–2 μM for μ-calpain and from 400–800 to 50–150 μM for m-calpain [4–7] with little or no change in specific activity of the enzymes [2,6]. Several kinetic studies found that autolysis closely parallels the onset of proteolytic activity for both μ- [8–10] and m- [2,11,12] calpain. That assays of proteolytic activity were always accompanied by autolysis raised the possibility that the unautolyzed calpains were proenzymes similar to prothrombin and that they required autolysis for their activation [12–14]. Hence, the unautolyzed calpains were sometimes referred to as "proenzymes". Kinetic evidence from several other studies, however, indicated that both μ- [5,15] and m- [16,17] calpain are proteolytically active before autolysis. Oxidation of μ-calpain inhibits its proteolytic activity but does not prevent its autolysis, therefore dissociating the two events in μ-calpain [18], and E-64, a covalent inhibitor of the calpains, reacted with the active site of m-calpain before any autolysis occurred indicating that the catalytic site was functional before autolysis [19]. Finally, sodium dodecyl sulfate poly-

Abbreviations: μ-calpain, the micromolar Ca^{2+}-requiring proteinase; m-calpain, the millimolar Ca^{2+}-requiring protease; IOV, inside-out vesicles; MCE, 2-mercaptoethanol; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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acrylamide gel electrophoresis (SDS-PAGE) results show that a casein substrate is cleaved to small fragments by either unautolyzed μ-calpain or unautolyzed m-calpain [20].

Although the unautolyzed calpains evidently are capable of proteolytic activity, the calpains are autolyzed under conditions where it can be demonstrated that they have been proteolytically active. This is especially evident during platelet activation [21–23] but is also observed in other cell types [24]. The Ca^{2+} concentrations required to initiate autolysis are as high as or even slightly higher than those required for proteolytic activity [5,25,26] and are much greater than the 40–500 nM free Ca^{2+} that is found in living cells [27–30] or even the 300–800 nM Ca^{2+} "spikes" found in stimulated cells [27,29]. A possible solution to this high Ca^{2+} requirement appeared when it was discovered that phospholipids [31], with phosphatidylinositol (PI) and phosphatidylinositol-4,5-bisphosphate (PIP_2) being the most effective [32,33], lowered the Ca^{2+} concentration required for autolysis from 550–800 to 90–400 μM for m-calpain and from 50–150 to 0.5–50 μM for μ-calpain [7]. Consequently, it was proposed that unautolyzed calpains are translocated to the plasma membrane in cells where interaction with a phospholipid moiety might lower the Ca^{2+} requirement for the autolysis sufficiently to enable their autolysis at intracellular free Ca^{2+} concentrations.

The membrane activation hypothesis has received widespread support, and a number of studies have examined binding of phospholipids to the calpains [34] and the effects of phospholipid on the Ca^{2+} concentration required for calpain autolysis [2,5,31–33]. It is surprising, however, that there have been no studies on the effects of plasma membranes themselves on the Ca^{2+} concentration required for autolysis of the calpains. Also, autolysis of a membrane-bound calpain implies that autolysis is an intramolecular process because only the membrane-bound calpain would have the reduced Ca^{2+} concentration required for autolysis. Even if membrane association was transient, the released calpain would need to have been "imprinted" by its association with the membrane to retain any effect that the membrane association had on [Ca^{2+}] required for catalysis. Such "imprinting" could involve a membrane-catalyzed phosphorylation/dephosphorylation event, but there has never been any evidence of such imprinting in cells. Indeed, autolysis of membrane-bound calpain is shown as an intramolecular event in schematic diagrams. A number of studies, however, have shown that autolysis of both μ- [10,20,35,36] and m- [1,11,20,37,38] calpain is an intermolecular process in in vitro assays. The nature of calpain autolysis in the presence of phospholipids has not been studied.

Therefore, we have compared the effect of PI and erythrocyte inside-out vesicles (IOV) on both the nature of calpain autolysis and on the Ca^{2+} concentration required for autolysis in an in vitro assay. The results show that autolysis of both μ- and m-calpain is an intermolecular process in the presence of PI and that IOV have no effect on the Ca^{2+} concentration required for autolysis of the calpains.

2. Materials and methods

2.1. Materials

Acrylamide (99.9%), bisacrylamide (99.99%), and sodium dodecyl sulfate (99%) were all from Swartz/Mann Biotech, Cleveland, OH; Tris (ultrapure, 99.8%) was from Amresco, Solon, OH; EDTA (free acid, 99%), L-α-PI and β-casein were purchased from Sigma Chemical Co. All other chemicals were reagent grade or purer.

2.2. Effect of PI on autolysis

All studies in this paper used μ- and m-calpain purified from bovine skeletal muscle by using the procedures that we have described [6,39]. For experiments using SDS-PAGE to test the effects of PI on calpain autolysis, the final conditions for autolysis were: 100 mM KCl; 50 mM Tris–HCl, pH 7.5; 20 mM MCE; 1.0 mM EDTA; 0 or 50 μM PI (a molecular mass of 902 Da was used for PI); 0 or 107.6 μg β-casein/ml (μ-calpain) or 165 μg β-casein/ml (m-calpain); 100 μg μ-calpain/ml or 150 μg m-calpain/ml; sufficient $CaCl_2$ to give 80 μM free Ca^{2+} (μ-calpain) or 400 μM free Ca^{2+} (m-calpain) in 100-μl final volume at 25.0 °C and for the times specified. Tubes containing all the ingredients except Ca^{2+} were incubated at 25.0 °C for 5 min, and autolysis was initiated by adding the designated amount of Ca^{2+}. After specified times, autolysis was stopped by adding 10 μl of 12 mM EDTA. Fifty-five microliters of SDS tracking dye was added to the 110 μl in the reaction tube, and the samples were subjected to SDS-PAGE. The quantities of β-casein added were sufficient to produce a calpain to β-casein molar ratio of 1:5 (molecular mass of β-casein is 23.6 kDa calculated from its amino acid sequence).

PI vesicles were prepared by dissolving PI in chloroform, the solution was dried in a Speed-vac, and the dried preparation was stored under argon gas at −85 °C until used. An aliquot was suspended in Na phosphate buffer at pH 7.0, the suspension sonicated for 30 min, and the sonicated vesicles used in the experiments. Formation of vesicles was verified by using HPLC size-exclusion chromatography.

SDS-PAGE was done by using the Laemmli system [40] as described previously [41]. Gels were 12.5% or 15% polyacrylamide containing 2.66% bis-acrylamide as percent of the acrylamide and were 1.5-mm thick and 14 × 16 cm to increase the distance between closely spaced bands and provide for more accurate densitometry. All gels were stained with Coomassie brilliant blue R250 and were destained by diffusion for 24–36 h [41] before scanning. Rate of autolysis of the calpains was determined by scanning the SDS-polyacrylamide gels with a Molecular Dynamics 300A computing densitometer and measuring the rate of disappearance of the 28- and 80-kDa polypeptide subunits of the unautolyzed calpains. This densitometer could resolve the 78- and 76-kDa polypeptides produced during the first few minutes of autolysis of the 80-kDa subunit μ-calpain

[26,35] if small quantities of protein were loaded on the gel, but it could not resolve these polypeptides at protein loads sufficiently high to allow monitoring of the disappearance of the 28-kDa subunit on the same gel. Similarly, the densitometer could not resolve the 78-kDa polypeptide produced by the first autolytic cleavage of m-calpain [16] from the unautolyzed 80-kDa subunit at protein loads sufficiently high to allow measurement of disappearance of the 28-kDa subunit in the same scan. Therefore, the scans measured disappearance of the 80- and 28-kDa subunits of μ-calpain and of the 78/80-kDa complex and the 28-kDa subunit of m-calpain. We did not attempt to determine the absolute concentration of the polypeptides in the gels that were scanned. Disappearance of the 80-kDa or the 28-kDa subunits was compared with a scan of a control, unautolyzed calpain having intact 28- and 80-kDa polypeptides on the same gel, so any differences in the degree of staining or destaining among the different gels did not affect the results.

The complications involved in using densitometry to measure rates of autolysis of the calpains have been discussed [20]. The goal of the work described in this paper was to determine whether the presence of PI changed the mechanism of autolysis of μ- and m-calpain. The simplest way to accomplish this was to determine whether the presence of a readily degraded substrate such as casein would alter the rate of autolysis. If autolysis is an intermolecular process, the presence of a substrate such as casein with a low K_m for the calpains would be expected to compete with the calpain molecules for proteolytic degradation and thus decrease the rate of autolysis. The presence of substrate should have no effect on the rate of intramolecular autolysis [20]. Consequently, the study was limited to learning whether the presence of β-casein would alter the rates of autolysis of the two subunits of μ- and m-calpain.

2.3. Effect of PI and IOV on the [Ca^{2+}] required for autolysis

For experiments studying the effect of IOV and PI on the Ca^{2+} concentration required for autolysis, IOV were prepared from dated human erythrocytes by using the procedure described by Steck and Kant [42]. The sidedness of the vesicles was determined by assaying for acetylcholinesterase activity in the presence or absence of 0.2% Triton X-100. Approximately 50–55% of total vesicles were inside-out. Phase microscopy confirmed that the preparations were almost entirely vesicles. The vesicles were suspended in 0.5 mM Tris–HCl pH 8.0, 0.1 mM EDTA, 1.0 mM NaN_3, and were stored at 0 °C until used; the vesicles were used within 5 days after their preparation. The effect of IOV on the Ca^{2+} concentration required for autolysis was measured by using the approach described by Cong et al. [5] in which the calpains are incubated at different free Ca^{2+} concentrations in the absence or presence of IOV, and the amount of autolysis that occurs during this incubation is determined by assaying for calpain activity at a Ca^{2+} concentration where only the autolyzed calpain is proteolytically active (12 μM for μ-calpain and 130 μM for m-calpain; although these concentrations are at the upper end of the reported range of Ca^{2+} concentrations required for proteolytic activity of the autolyzed calpains, they were selected on the basis of preliminary experiments in which activities of the autolyzed and unautolyzed forms of the calpains were assayed at different free Ca^{2+} concentrations).

Final conditions of the incubation to induce autolysis in the presence of IOV or PI were: 100 mM KCl; 50 mM Tris–HCl, pH 7.5; 1.0 mM EDTA; 20 mM MCE; with or without 1000 μg IOV protein/ml or 50 μM PI as indicated; 100 μg μ- or m-calpain/ml; and different $CaCl_2$ amounts to produce the specified free Ca^{2+} in the assay. Assuming that 52% of the erythrocyte membrane mass is protein and 40% is lipid [43] and that approximately 40% of the total lipid in erythrocyte ghosts is on the extracellular surface [44], 1000-μg IOV protein would contain approximately 150 μg of "intracellular" phospholipid if 50% of the vesicles were inside-out. Hence, the phospholipid content of the IOV is similar to the PI concentrations used in our studies. Tubes containing all the ingredients except Ca^{2+} were incubated for 5 min at 25 °C, and autolysis was initiated by the addition of Ca^{2+}. Autolysis was done for 2 min at 25.0 °C and was stopped by adding EDTA to a final concentration of 5 mM. Aliquots of the autolyzed samples were removed and were assayed for proteolytic activity by using the FITC-casein fluorescence assay [41]. Final conditions for the assay of proteolytic activity were: 2.5 mg FITC-casein/ml; 100 mM KCl; 20 mM Tris–HCl, pH 7.5; 2.6 mM EDTA; 12 (μ-calpain) or 130 (m-calpain) μM free Ca^{2+}; 20 mM MCE; 364 μg IOV/ml or 18 μM PI; 36.4 μg μ- or m-calpain/ml for 30 min at 25 °C.

Several experiments were done to determine whether the calpains bound to the IOV under the conditions used in this study. Samples of μ- or m-calpain were mixed with IOV in 100 mM KCl; 50 mM Tris–HCl, pH 7.5; 1.0 mM EDTA; and 20 mM MCE. After 5 min at 25 °C, Ca^{2+} was added to produce a free Ca^{2+} concentration of 0.5 μM (μ-calpain) or 500 μM (m-calpain) and the mixture was incubated for 1 min before an aliquot was removed for calpain assay. In some experiments, 100 μM leupeptin was added to suppress autolysis, although autolysis was negligible at the short times (1 min) and Ca^{2+} concentrations that we used. Fig. 1 confirms that very little autolysis occurs during the first minute under similar conditions. The remaining suspension was sedimented at $55{,}000 \times g_{max}$, and the sedimented pellet was washed and the combined supernatants were dialyzed and were assayed for calpain activity. The calpain concentration in these studies was 50 or 100 μg/ml and the IOV concentration varied from 12.5 to 200 μg protein/100 μl.

EDTA (and EGTA) is a very poor Ca^{2+} buffer in the free Ca^{2+} concentration range from 0.5 to 100 μM (see Ref. [7] for a plot of free Ca^{2+} vs. total Ca^{2+} in the presence of 1 mM or 5 mM EDTA). Hence, the actual free [Ca^{2+}] in the

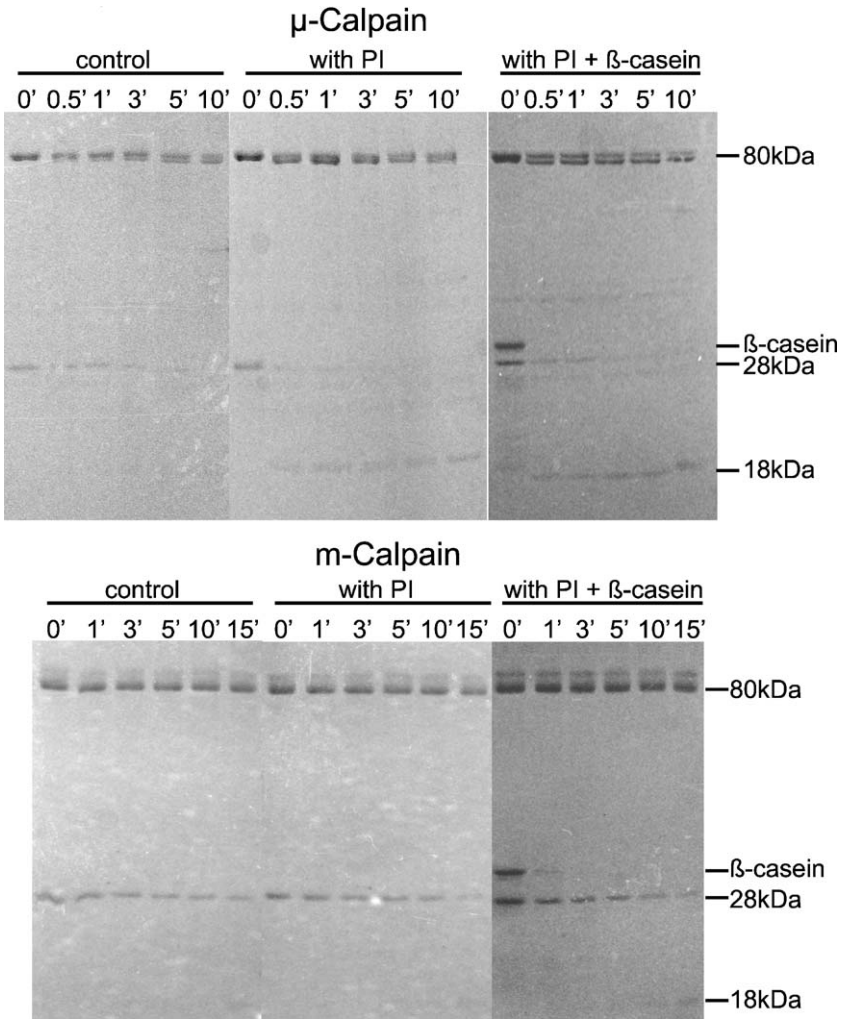

Fig. 1. SDS-polyacrylamide gels showing the time course of autolysis of μ- and m-calpain in the presence or absence of PI and in the presence of both PI and a fivefold molar excess of β-casein. Conditions: Autolysis was done as described in Materials and methods at a free Ca^{2+} concentration of 80 μM (μ-calpain) or 400 μM (m-calpain). A 15% polyacrylamide gel was used in the μ-calpain experiment, and a 12.5% polyacrylamide gel was used in the m-calpain experiment. Times of autolysis are indicated above each lane. The lanes were loaded with 3-μg protein (μ-calpain) or 2.5-μg protein (m-calpain). 0 is a sample in which EDTA was added before the Ca^{2+}. The diffuse band above the 80-kDa subunit of m-calpain resulted from the electrophoresis procedure used in this experiment and had no effect on the rate of autolysis. The β-casein (mass of 23.6 kDa) migrates anomalously at approximately 32 kDa just above the 28-kDa subunit. These gels are examples of the gels that were scanned to produce the data in Figs. 2 and 3.

0.5 μM Ca^{2+} buffers may have ranged from 0.2–0.3 to as high as 50 μM (buffering capacity increases markedly as free [Ca^{2+}] decreases below 0.3 μM or increases above 100 μM; Ref. [7]). This problem exists only in the range 0.5 to 100 μM and is not solved by measuring free Ca^{2+} with a Ca^{2+} dye, for example, because the dye also binds Ca^{2+} in making the measurement, and the difficulty lies in the poor buffering capacity. The problem is ameliorated somewhat by using higher EDTA concentrations (e.g., 1 or 2.6 mM in the present study instead of 0.1 mM). The poor Ca^{2+} buffering capacity did not impact the results in the present study because: (1) calpain bound to the IOV at the nominal 0.5 μM Ca^{2+} used; the buffering capacity of EDTA increases below 0.2–0.3 μM free Ca^{2+}, so it is very unlikely that the actual free Ca^{2+} would have fallen below these values; an increase up to 50 μM, if it had happened, would have increased the amount of calpain binding to the IOV and would also have increased the rate/extent of autolysis, neither of which was observed; and (2) if free [Ca^{2+}] had deceased below the nominal 12 μM in the assays for activity of autolyzed μ-calpain, no proteolytic activity would have been detected, whereas we did detect such activity; and if the actual free Ca^{2+} increased to 80–100 μM, μ-calpain activity would have been detected at far lower [Ca^{2+}] than what was observed (Fig. 4). The use of 1 mM EDTA concentrations also decreases any differential effects that Ca^{2+} binding to IOV or PI might have had on the results.

2.4. Other methods

Protein concentration was determined by using the bicinchoninic acid procedure [45] and bovine serum albumin

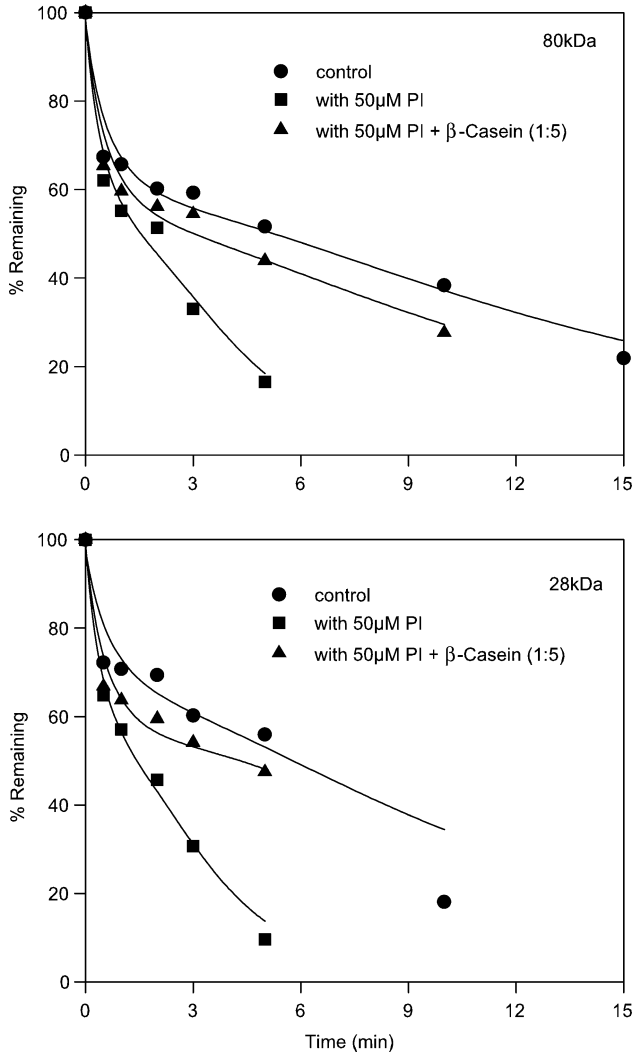

Fig. 2. Effect of PI or PI plus β-casein on the rate of autolysis of μ-calpain. Conditions: Autolysis was done as described in Materials and methods at 80 μM free Ca^{2+} and without PI, with PI, or with PI plus a 1:5 molar ratio of μ-calpain/β-casein. Gels were scanned with a densitometer as described in Materials and Methods. The plots in this figure and in Fig. 3 were obtained by using the SigmaPlot and TableCurve programs (SPSS, Chicago, IL). The 10-min value for autolysis of the Control 28-kDa subunit (lower panel) was much below the curve fitted by the computer program. Such a low value was not generally observed and was unique to this experiment.

calibrated by Kjeldahl nitrogen analysis to prepare the standard curves. All experiments used reverse-osmosis purified water that had been passed through successive deionizing cartridges, then through an organic filter, and finally through a microfiltration filter. This water was further purified by using a Millipore Milli-Q-Plus filtration system before preparing solutions of specified free Ca^{2+} concentrations. The EqCal program (Biosoft, Milltown, NJ) was used to calculate free Ca^{2+} concentrations in our Ca^{2+}-buffers and assays [46]. The actual Ca^{2+} concentrations of the stock solutions used to prepare the buffers were measured with atomic absorption spectroscopy.

3. Results

3.1. Autolysis of μ- and m-calpain in the presence of β-casein and PI

The experiments in this study were done at Ca^{2+} concentrations lower than those required for a maximum rate of autolysis [7], so the ability of PI to reduce the Ca^{2+} concentration needed for autolysis is observed as an increased rate of autolysis in the presence of PI (Fig. 1). The rate of autolysis of μ-calpain is much faster than the rate of autolysis of m-calpain at the Ca^{2+} concentrations used in Figs. 1–3 (compare rate of disappearance of the 28-kDa subunit in Figs. 2 and 3). In agreement with earlier studies [5,17,31–33], the presence of 50 μM PI significantly reduces the Ca^{2+} concentration required for autolysis (observed as an increase in rate of autolysis under our exper-

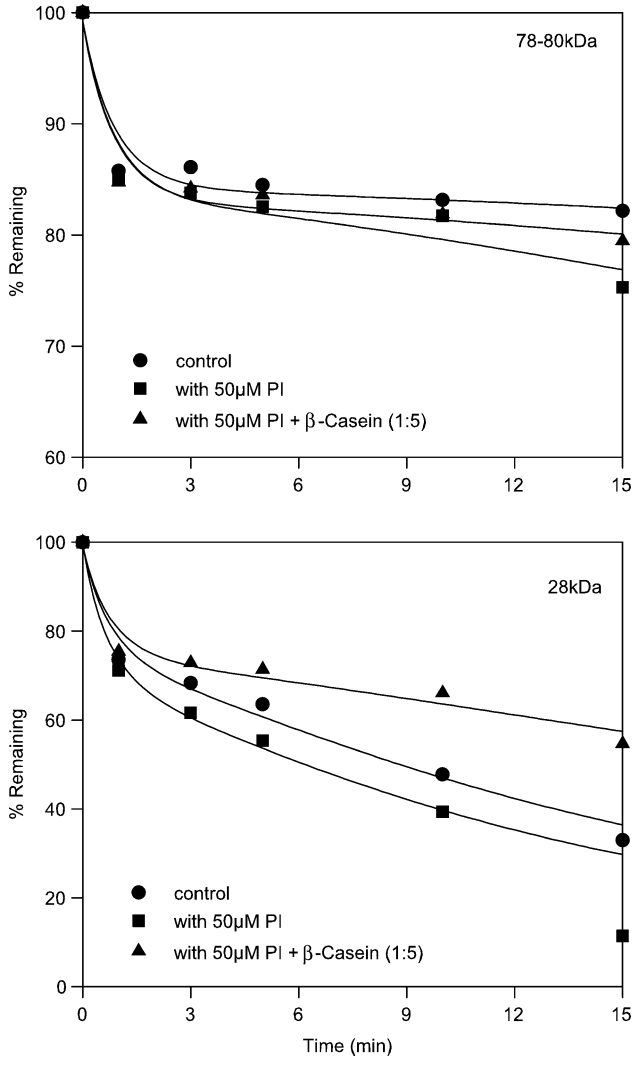

Fig. 3. Effect of PI or PI plus β-casein on rate of autolysis of m-calpain. Conditions: Autolysis was done as described in Materials and Methods at 300 μM free Ca^{2+} and without PI, with PI, or with PI plus a 1:5 molar ratio of m-calpain/β-casein. Gels were scanned with a densitometer as described in Materials and methods.

imental conditions) of both μ- and m-calpain, even in the presence of β-casein (Figs. 1–3). That a fivefold molar excess of a digestible substrate, β-casein, significantly decreases the rate of μ- and m-calpain autolysis (most easily observed in the rate of disappearance of the 28-kDa subunits in Figs. 2 and 3) indicates that autolysis of both calpains is an intermolecular process at the conditions used in these experiments, and that the presence of PI does not change this intermolecular mechanism to an intramolecular mechanism as is commonly depicted in schematic diagrams of membrane activation of the calpains.

3.2. Effect of IOV on Ca^{2+} concentration required for autolysis of μ- and m-calpain

IOV have no effect on the Ca^{2+} concentration required for autolysis of either μ- or m-calpain (Figs. 4 and 5). Addition of PI to the same enzymes assayed under the same conditions, however, significantly reduces the Ca^{2+} concentration required for autolysis (Figs. 4 and 5). Consequently, the calpains used in these experiments were capable of responding in the usual way to PI. The effect of IOV prepared from human erythrocytes on the Ca^{2+} concentration required for autolysis of μ-calpain also prepared from human erythrocytes was assayed to learn whether any effect of cell membranes on the Ca^{2+} concentration required for autolysis might be specific for the calpain from that cell. Erythrocyte IOV had no effect on the Ca^{2+} concentration required for autolysis of erythrocyte μ-calpain either, indicating that the results in Figs. 4 and 5 were not caused by the use of bovine skeletal muscle calpains in these studies (results not shown here).

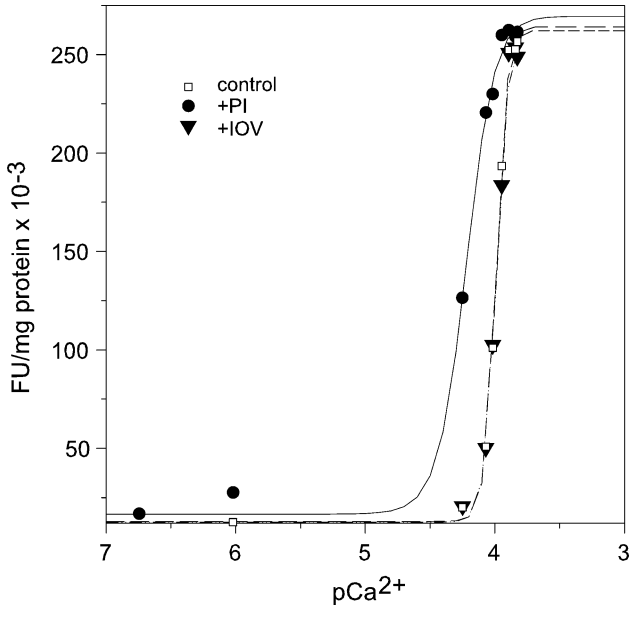

Fig. 4. Effect of IOV or PI on the Ca^{2+} concentration required for autolysis of μ-calpain. After autolysis for 2 min at the free Ca^{2+} concentrations indicated, proteolytic activity of the μ-calpain was assayed at 12 μM free Ca^{2+}, a Ca^{2+} concentration where autolyzed μ-calpain is active but unautolyzed μ-calpain is barely active. The Ca^{2+} concentrations required for half-maximal autolysis as measured by this procedure are: 60.3 μM in the presence of PI; 103 μM in the presence of IOV, and 102 μM in the absence of either IOV or PI. The plots and Ca^{2+} concentrations required for half-maximal autolysis in this figure and in Fig. 5 were obtained by using the SigmaPlot and TableCurve programs.

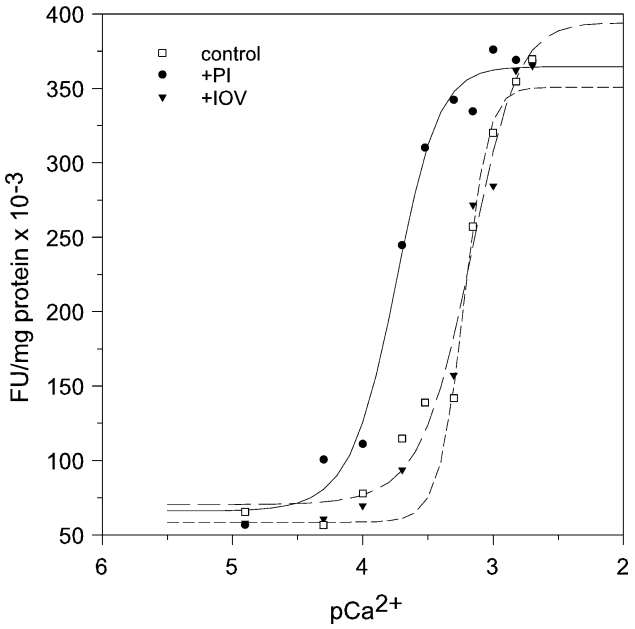

Fig. 5. Effect of IOV or PI on the Ca^{2+} concentration required for autolysis of m-calpain. After autolysis for 2 min at the Ca^{2+} concentrations indicated, proteolytic activity was assayed at 130 μM free Ca^{2+}, a Ca^{2+} concentration where autolyzed m-calpain is active but unautolyzed m-calpain is barely active. The Ca^{2+} concentrations required for half-maximal autolysis as measured by using this procedure are: 176 μM in the presence of PI, 597 μM in the presence of IOV, and 658 μM in the absence of either PI or IOV.

Several studies were done to show that μ- and m-calpain bound to the IOV used in this study. Increasing the amount of IOV protein from 12.5 to 200 μg/100 μl resulted in a 3.5- to 4-fold increase in the amount of μ- and m-calpain bound to the IOV, respectively. Simultaneously, the amount of μ- or m-calpain activity in the supernatant remaining after sedimentation of the IOV decreased. We did not observe saturable binding under the conditions that we used (100 μg calpain/ml and Ca^{2+} concentrations of 0.5 or 500 μM). The calpains do not precipitate at Ca^{2+} concentrations below 800 μM at pH 7.5, so it is unlikely that the results were due to precipitation of the calpains that was interpreted as "binding". A number of studies have found that the calpains bind to IOV, although these studies did not determine whether the binding was saturable [47–50]. Because the calpains bound to IOV at the conditions we used, the lack of effect of IOV on the Ca^{2+} concentration required for autolysis is not due to lack of binding of the IOV to the calpains.

4. Discussion

Although it has widely been assumed that the ability of a phospholipid such as PI or PIP_2 to reduce the Ca^{2+}

concentration required for autolysis of the calpains in in vitro assays can be extrapolated to interaction with a PI or PIP_2 moiety in the plasma membrane, there have been no studies to show whether plasma membranes actually affect the Ca^{2+} concentration required for autolysis. The present study shows that IOV from erythrocytes have no effect on the Ca^{2+} concentration required for autolysis of either μ- or m-calpain, even though PI did lower the Ca^{2+} concentration required for autolysis of the same calpains. Although PIP_2 lowers the Ca^{2+} concentration required for autolysis of μ-calpain to approximately 100 nM [32,33], whereas PI lowers it to approximately 1000 nM (32, 33) to 1400–1500 μM [5], both PIP_2 and PI have the same effect on autolysis at higher Ca^{2+} concentrations (above 10 μM), suggesting that the difference is one of potency rather than a fundamental difference in mechanism [32,33]. There are several conceptual difficulties in directly extrapolating the effects of PI or PIP_2 on autolysis of the calpains in in vitro assays to supposing that binding of the calpains to plasma membranes would have the same effect in lowering the Ca^{2+} concentration required for their autolysis to physiological Ca^{2+} concentrations. First, although the Ca^{2+} concentration required for autolysis of μ-calpain in the presence of PIP_2 [32,33] is only slightly greater than the free Ca^{2+} concentration found in cells, the Ca^{2+} concentration required for autolysis of m-calpain even in the presence of PIP_2 is still an order of magnitude higher than that found in living cells [2,5,7]. Hence, some other mechanism would have to be involved in activation of m-calpain. Second, the PIP_2 concentration required to reduce the Ca^{2+} concentration required for autolysis of either μ- or m-calpain was 29 (for PIP_2; Ref. 33) to 75 (for PI; Ref. 31) μM, which is approximately a 2500:1 to 400:1 molar ratio of PIP_2 or PI, respectively, to the calpain concentration used in the assays. Consequently, the PIP_2 or PI/calpain interaction in these in vitro assays was not a stoichiometric one but likely involved interaction of the calpain molecule with a phospholipid micelle [17]. It is unclear how such an interaction relates to interaction with a PIP_2 (or PI) moiety in a plasma membrane. Phospholipid concentrations lower than 7.54 μM (μ-calpain; PIP_2/μ-calpain molar ratio of 650; Ref. 33) or 5.5 μM (m-calpain; PI/m-calpain molar ratios of 28:1; Ref. 2) had almost no effect on the rate of autolysis in in vitro assays, indicating that interaction with a single PIP_2 molecule would have little or no effect on the Ca^{2+} concentration required for autolysis of the calpains. Third, it is not certain that plasma membranes contain sufficient PI or PIP_2 to act as efficient activators of the calpains. Erythrocyte membranes, as an example, are approximately 50% protein and 40% lipid [43,51,52]; of this lipid, approximately 60–70% is phospholipid [52,53], but PI and PIP_2 together constitute less than 5% of this phospholipid (less than 2% of the total erythrocyte, 54). Although this PI and PIP_2 is almost entirely on the intracellular surface of the membrane, only 46–60% of it is accessible to phosphatidylinositol/phosphatidylinositol-4-phosphate kinase [55]. Presumably, this "masked" PI/PIP_2 would also be unavailable for interacting with calpain. Much of the PI that is inaccessible to phosphatidylinositol/phosphatidyl-4-phosphate kinase in the membrane may be in the form of glycosyl phosphatidylinositol anchors. Hence, there are few PI or PIP_2 sites on erythrocyte plasma membranes and only a small percentage of them are available for binding the calpains. Although plasma membranes from other cell types have different lipid compositions, PI and PIP_2 in general are not abundant components of plasma membranes. Phosphatidylserine is present on the intracellular surface and is a more abundant component of the plasma membrane than PI or PIP_2. Phosphatidylserine, however, has little effect on the Ca^{2+} concentration required for calpain autolysis in in vitro assays [32,33]. Fourth and finally, several studies have reported that the calpains do not bind to phospholipids in the plasma membrane but to protein molecules instead [47–50].

Despite these concerns, studies have found that association of the calpains with the plasma membrane, either in intact cells [22,23,35,56] or in in vitro assays [25], is often accompanied by their autolysis. These studies, however, did not show whether membrane association lowers the Ca^{2+} requirement for autolysis, as is usually assumed, or whether some other event initiates autolysis [57]. The resolution of these studies also did not eliminate the possibility that the autolysis occurs *before* the calpains actually bind to the membrane. Indeed, it has been reported that μ-calpain in human platelets undergoes autolysis in the cytoplasm and that the autolyzed molecules subsequently bind to the plasma membrane [58]. We (Thompson, Li, and Goll, unpublished) have found that autolysis increases the hydrophobicity of both μ- and m-calpain, so that they cannot be eluted from phenyl Sepharose hydrophobic columns unless SDS is used. One possible function of autolysis, therefore, may be to convert the calpains into hydrophobic molecules that bind to their substrates in the plasma membrane and elsewhere. Such a function would explain the prevalence of autolyzed calpains at the plasma membrane. That several studies have found that the calpains bind to proteins and not to phospholipids in erythrocyte membranes [48–50] also is consistent with this concept.

Consequently, when examined closely, it seems unlikely that interaction with a PI or PIP_2 moiety in the plasma membrane reduces the Ca^{2+} concentration required for autolysis of the calpains in vivo. Plasma membranes in living cells are dynamic structures, and it is not clear how well the IOV used in this and other studies [25,47–50] mimic the properties of plasma membranes that undergo ruffling, invagination, etc., in living cells. The presence of autolyzed calpains at the plasma membrane may involve interactions and events such as tyrosine phosphorylation that might initiate autolysis and that have not yet been considered.

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